MOLECULAR DETECTION OF *MYCOBACTERIUM AVIUM* subsp. *PARATUBERCULOSIS*

Field of the Invention

The present invention relates to the molecular detection of the *Mycobacterium avium* subsp. *paratuberculosis (MAP)*. More particularly it relates to PCR assays with pairs of oligonucleotide primers targeted to different genomic regions of the IS900 element specific for MAP, for use in the molecular detection and identification of MAP.

Prior art

The *Mycobacterium avium* subsp. *paratuberculosis (MAP)* is a very fastidious bacterium from the point of view of microbiological identification, that can cause a serious and often fatal disease, namely paratuberculosis, to several animal species. Furthermore, an increasing number of reports are implicating the specific bacteria *MAP* to the pathogenesis of certain forms of Infectious Bowel Disease of man (Chiodini, 1989), (Chamberlin et al., 2001).

Identification of *MAP* is nowadays facilitated by the broad application of molecular techniques. However, the need for verification of results often makes existing techniques laborious and practically applicable only for research use.

More specifically, *MAP* infections' diagnosis obtained by serology requires further confirmation, in order to avoid false-negative results that are often recorded at the early stages of infection and cross-reactions with genetically related mycobacterial species found in nature (Ridge, et al. 1991; Nielsen, et al. 2001). The usefulness of culture, as a diagnostic or confirmatory method, is impaired by the long incubation period required for the growth of *MAP*.

Thus, the Polymerase Chain Reaction (PCR) has long been considered a very useful tool for the detection and identification of the above bacterium (Hance et al., 1989). However, confirmation of results is necessary also for molecular techniques, especially when they are applied for routine diagnosis. This usually suggests the incorporation of hybridization assays, which however make the entire process laborious and practically applicable only for research use.

Thus, a low-cost and rapid PCR-based method that could be incorporated reliably to the routine identification of MAP from clinical material is of considerable significance. Low cost must be combined with very high sensitivity and specificity. It is also very important to secure that the results may be repeated very often, especially because it is often found in

practice that methods that function satisfactorily in a laboratory, when used in another laboratory may give results that cannot be used.

Summary of the Invention

The present invention has enabled the development of a low-cost and rapid PCR-based method that can be incorporated reliably to the routine identification of *MAP* from clinical or from any other material.

In order to secure the reliability of the proposed assay and the reproducibility of the results in different laboratories, intra-laboratory crosschecking has taken place.

The invention made it possible to develop a method for the routine detection and identification of *MAP*, from tissue samples of human, animal, vegetal or other origin. The said method may be applied not only on clinical material, but also on other material of human, animal or plant origin, on dust, on food products in general etc.

The proposed assay combines optimum performance and cost with high reproducibility, regardless the various specific conditions and practices and it is applicable in different laboratories.

The invention is realized through the development of a PCR assay using one or more of the oligonucleotide primers that are described below:

P1N	GCATGGCCCACAGGACGTTGAG
P2N	CTACAACAAGAGCCGTGCCG
P3N	GGGTGTGGCGTTTTCCTTCG
P4N	TCCTGGGCGCTGAGTTCCTC

In a preferred embodiment combinations of the oligonucleotide primers, in order to form sets of oligonucleotides, are used. Preferred sets of oligonucleotides are P1N/P3N, P1N/P4N, P2N/P3N, P2N/P4N, P1N/P2N/P3N, P1N/P2N/P4N, P1N/P3N/P4N, P2N/P3N/P4N. Particularly preferred is the set of oligonucleotides comprising all four oligonucleotide primers, namely P1N/P2N/P3N/P4N.

These primers are targeted to genomic region of the IS900 element specific for *MAP* and enable the effective, fast and reliable detection of *Mycobacterium avium* subsp.

paratuberculosis (MAP). These primers were selected among more because they have better results.

Detailed description

The use of the above mentioned oligonucleotide primers – alone or in combination - were found to be preferable in a one-tube PCR assay for the reliable detection of the MAP.

In a preferred embodiment combinations of the oligonucleotide primers, in order to form sets of oligonucleotides, are used. The sets of oligonucleotides useful for the detection of *MAP* are P1N/P2N, P3N/P4N, P1N/P3N, P1N/P4N, P2N/P3N, P2N/P4N, P1N/P2N/P3N, P1N/P2N/P4N, P1N/P3N/P4N, P2N/P3N/P4N.

In a further and particularly preferred embodiment the set of oligonucleotides comprising all four oligonucleotide primers, namely P1N/P2N/P3N/P4N, is used. Further preferred is the use of a one-tube nested PCR, using all four oligonucleotide primers, namely P1N/P2N/P3N/P4N.

In order to reach a method for the reliable detection of *MAP* by PCR, tests were conducted in several laboratories, some of which were even in different countries. In order to detect the exactitude and the reliability of the method, all the different parameters that normally compose a PCR reaction were not harmonized and this factor was also incorporated for the assessment of our method. The above approach has never been described before with reference to *MAP*.

Different laboratories undertook to evaluate the DNA extraction procedures and the different PCR assays for the detection of *MAP*. For DNA extraction one in-house, and one commercial method were used, whereas for PCR a significant number of different assays were assessed, starting with the evaluation of primer specificity with extended GenBank database search.

Thus, we concluded to a one tube-nested, a two nested, and a single, PCR assay, targeted to different genomic regions of the IS900 element specific for *MAP*. These four methods were applied on positive and negative control samples, consisted of pure bacterial cultures and formalin-fixed paraffin embedded tissue samples (FFPE) collected from cattle with paratuberculosis and chickens with *Mycobacterium avium* infections. In the tests carried out all the samples were identified with coded numbers and the results were communicated among the collaborating laboratories. Based on the criteria of reliability and cost, the procedure that performed better was the one-tube nested PCR assay that uses the oligonucleotide primers mentioned above, combined with the in-house DNA extraction

method. This method produced the expected result from the positive and negative control samples used, allowing differentiation even among genetically related mycobacterial species. The agreement of the results obtained by the different laboratories where tests were performed indicates the reliability of the proposed assay even under different laboratory conditions.

Examples

Materials:

DNA was extracted from cultures and from clinical material (formalin-fixed paraffin embedded tissue samples (FFPE)) and the molecular techniques described below were performed on those.

In more detail, cultures of 10 strains of *Mycobacterium avium subsp. paratuberculosis* (*MAP*) and 30 strains of various other members of the *Mycobacterium* spp. were utilized (as described in detail in Table 1). For the evaluation of specificity of the method described, cultures of several genetically related bacterial species (*Escherichia coli*, *Staphylococcus aureus*, *Salmonella* spp., *Enterobacter* spp.) were used.

The clinical material (tissue samples) that was included in the tests performed consisted of formalin fixed paraffin embedded (FFPE) samples collected from cattle and chickens. 10 intestine FFPE samples from cattle, with typical lesions of paratuberculosis were used. An equal number of liver and intestine, FFPE samples from chickens, bearing typical lesions of mycobacterial infection were used. Corresponding clinical material from the cattle and the chickens was positive by culture for *MAP* and *Mycobacterium avium subsp. avium (MAA)* respectively. Additionally 30 fresh and FFPE, intestine and lymph node samples, collected from humans and animals with no clinical or other indication of mycobacterial infection were examined. This material was used as negative control (this data is not shown in the present specification).

Methods:

A) DNA extraction from FFPE tissue samples and bacterial cultures.

DNA extraction from the FFPE samples was performed in two ways, both used on 2 to 3 paraffin sections 10μm-thick per sample:

A1. For DNA extraction, carried out as previously described (in-house method) (Ikonomopoulos et al., 1999), the material was dewaxed by repeated incubations in xylene at 60°C, and ethanol 100% and finally ethanol 75%. The product of the above process is

incubated for approximately one hour in SDS and proteinase K in 50 ^{0}C temperature so that it is subsequently digested.

These proteins were removed with the addition successively of phenol and phenol – chloroform - isoamyl alcohol solution. Subsequently, the DNA is precipitated ethanol and natrium acetate (Sambrook et al., 1989) and is collected in form of sediment which is then eluted in 50μl of TE buffer.

A2. Alternatively, DNA extraction from FFPE samples was performed with the MicroLysis kit (Microzone) according to the manufacturer 's instructions.

DNA extraction from bacteria was performed with CTAB-proteinase K followed by a combination of phenol and phenol-chlorophorm-isoamyl alcohol. The DNA extract was precipitated by ethanol and natrium acetate as described above.

Evaluation of the quality of the DNA extract with reference to quantity, purity, and integrity was performed with optical density counts and agarose gel electrophoresis.

B) Polymerase Chain Reaction (PCR):

A significant number of PCR assays were assessed, starting with the evaluation of primer specificity with extended GenBank database search with NCBI BLAST. Thus, 4 different PCR assays with a total of 7 pairs of oligonucleotide primers, targeted to different genomic regions of the IS900 element specific for *MAP* were concluded to and assessed. Those different PCR assays are described below under B1, B2, B3 and B4. More specifically:

B1. The first control PCR assay was with the oligonucleotide primers IS900-F and IS900-R (shown in Table 2). These primers amplify a 707 bp DNA fragment of the IS900 element of *MAP* (Green et al 1989).

B2. Another control PCR assay was nested PCR assay with the primers s204 and s749 (Table 2) that amplify a 563 bp DNA fragment of the IS900 element of *MAP*. The product of this reaction diluted 1:10, is incorporated into the second reaction with the primers s345 and s535 (Table 2) that amplify a 210 bp DNA fragment within the 563 bp DNA fragment amplified by the s204-s749 primers (Englund et al 1999).

B3. Another control PCR assay was nested PCR assay with the primers IS01 and IS04 (Table 2) that amplify a 302 bp DNA fragment of the IS900 element. The product of this reaction diluted 1:10, is incorporated into the second reaction with the primers IS02 and IS03 (Table 2) that amplify a 159 bp DNA fragment within the 302 bp DNA fragment amplified previously.

B4. Finally, one PCR assay according to the invention was a one-tube nested PCR assay comprising the primers P1N, P2N, P3N and P4N (Table 2). All the primers are added simultaneously in the mixture of the PCR reaction that amplifies a 257 bp DNA fragment of the IS900 element.

The reaction mixture for each one of the PCR assays mentioned above was prepared with 50 mM KCl, 10mM Tris-HCl (pH 8,4 at room temperature), 1.5mM $MgCl_2$, 0.25μM of each of the oligonucleotide primers, 200μM dNTPs, 2.5 units Taq polymerase (Promega), 0.05-0.1 μg DNA, and HPLC quality water to a final volume of 50 μl for the assays B1 and B4, and 25 μl for the assays B2 and B3.

The temperature profile that was used for the assays B1-B3 (Table 3), consisted of an initial 5min denaturation step at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 63°C (for the assays B1 and B2) or 62 °C (for the assay B3) and 1 min at 72°C. This stage was followed by a 10-min incubation step at 72°C for the completion of the DNA product (Table 3). Both stages of the nested reactions B2 and B3, were performed at the same temperature profile as opposed to the assay B4 that involved different temperature profiles for each of the two stages of the nested reaction (Table 3). The first stage consisted of an initial denaturation step at 94°C for 1 min, followed by 16 cycles, with 1 min at 94 °C, 1 min at 54 °C, and 1 min at 72 °C, and 30 cycles with 1 min at 94 °C, 1 min at 67°C, and 1 min at 72 °C, followed by a final extension step of 3 min at 72 °C (Table 3). The PCR products were analysed by electrophoresis in 2% agarose gels, stained by ethidium bromide, and photographed.

The evaluation of the sensitivity of each of the above methods was determined as previously described (Ikonomopoulos et al., 1998) with their application on decimal dilutions of DNA extracted from *MAP* cultures.

Evaluation of reproducibility:

The DNA extraction methods and PCR assays were applied on the reference and clinical material described above, under the different specific conditions of each different laboratory (type of thermocycler, type of DNA-polymerase, buffers). The samples were identified with coded numbers and the results were communicated between the different laboratories and compared.

Results

A. DNA extraction:

The methods A1 and A2, produced DNA of satisfactory quality and quantity from the FFPE samples used. The amount of DNA produced by two to three 10μm-thick paraffin sections was estimated to 1μg/μl and its quality, with reference to purity and integrity, was the same regardless of the method that was utilized for the DNA extraction. However, the in-house method (A1), proved to be of considerably lower cost although more laborious.

The quality of the DNA product extracted from bacterial cultures with the in-house method (A1) was, as expected, very satisfactory with reference to quantity and integrity of the product.

B. PCR:

The application of the four PCR assays (B1, B2, B3 and B4) described above, on DNA extracted from the bacterial cultures used (Table 1), allowed the specific detection and identification of all the *MAP* strains that were utilized. On the other hand, none of the negative controls, both with reference to bacterial cultures and FFPE samples, produced positive results with any of the primer pairs that were evaluated. The number of positive results that were recorded with the methods B1, B2, B3 and B4, from the 10 FFPE samples derived from cattle with Johne 's disease, was 6, 9, 6, and 10, respectively (Table 4) (Figure 1).

The sensitivity of all the PCR methods that were evaluated was satisfactory, especially with reference to the nested assays. The minimum amount of DNA necessary for a positive result from bacterial cultures was estimated to 8 pg (method B4) corresponding to about 1500 bacterial cells (Baess et al., 1984).

C. Evaluation of reproducibility:

The application of the methods B1 to B4 to the detection of *MAP* from bacterial cultures and FFPE tissue samples produced completely consistent results in all the participating laboratories.

More specifically, it was found that methods B2 and B3 may cover diagnostically the whole range of MAP, only when used in combination, while the same problem was found also for method B1. Method B4 allowed the detection of MAP, regardless of the material used.

Based on our experimental data, it is shown that method B4, a one-tube nested PCR, that has at the same time the highest sensitivity, specificity and reproducibility, while at the

same time it enables directly to check the results in the least possible time and with the least necessary cost. It was found that methods B1 and B4 detected with specificity the *MAP*, among bacteria that was extracted from cultures. However, only methods B2 and B4 detected all the positive FFPE samples used. This, despite the fact that all primers were designed to detect MAP and their synthesis was defined based on the same genomic region (IS900). This fact indicates yet another parameter of the significance of the present invention, justifying the evaluation procedure that was designed, so that the method with the primers proposed by the present invention secures minimum false results for reliable routine application and not merely for research.

The discrepant results recorded with the methods B1 and B3 that were also verified by the intra-laboratory collaboration, were not surprising. The method B1 was expected to be less sensitive than the nested reactions, whereas the method B3 was designed specifically to implement and increase the diagnostic efficiency of the method B2. This was indeed proven accurate since the combination of the methods B2 and B3 did not identify correctly all our positive-control FFPE samples (Figure 1).

The discrepancies of the results recorded with the methods B1 and B3 could also be attributed to the genetic diversity of the *MAP* strains, at least with reference to the FFPE samples that were assessed. The presence of PCR inhibitors that can often lead to false negative results, especially with FFPE samples (Hermon-Taylor et al., 2000), cannot consist the cause of this discrepancy since the same DNA products were used for all the PCR reactions (also with the methods B2 and B4). For the same reason these results cannot be attributed to possible DNA fragmentation, since the product of the DNA extraction procedure was always assessed by electrophoresis and spectrophotometry and samples producing poor DNA quality were always discarded. Finally, the selection of the IS900 element as the common target of all the PCR reactions described above, should also be excluded from the factors that could result to the inefficiency of the methods B1 and B3. The selection of the certain DNA-target area was compulsory since the demonstration of the presence of the IS900 element is nowadays considered a prerequisite for the molecular identification of *MAP* (Green et al., 1998).

Method B4 presents important advantages compared also to method B2, irrespective of the fact that both methods are known to be diagnostically sufficient. More specifically, method B2 is a PCR assay comprising two consecutive stages. In order to proceed from one stage to the other, the tube that was used during the first stage must be opened and this is dangerous for infection, due to high possibility of entrance in the mix of the last reaction, of

DNA products from previous positive assays that are to be found a lot in the laboratory (carry-over effect). On the contrary, this fact, which often leads to the increase of the false positive results, is avoided by the method B4, since all the constituents of the nested assay are inserted together into the reaction tube and this is not opened during the assay. In addition to the above, method B4 has significant advantages compared to method B2, but also to method B3, because it requires significantly less time and less cost since less consumables are necessary as it involves one single reaction.

The above advantages of method B4 and the fact that, like for the other pre-mentioned PCR assays, the results are controlled without the need to use an internal control, which would significantly augment the cost and complexity of the assay, but also without the need for DNA hybridization, which among others would also augment the time needed for the procedure, constitute method B4 clearly superior to all the other methods that were assessed.

Besides the above, method B4 showed extremely high sensitivity, which makes it possible that the percentage of false negative results is extremely low. Further, the fact that the proposed assay (B4) detected exclusively only the *MAP* from all the materials used, shows its very high specificity, which also makes it possible that the percentage of false negative results is extremely low. In this respect, it is important to state that the said assay showed absolute specificity, not only with the bacteria that were extracted in culture. But also in FFPE samples originating from chicken infected by *Mycobacterium avium*, which presents especially high genetic relation to *MAP*. As opposed to the expected inefficiency of some of the other methods that were assessed, the complete reproducibility of the results that were recorded by the collaborating laboratories in respect of method B4, increases its reliability and supports the above.

In conclusion, it can be sustained that the nested PCR assay B4 is characterized by speed and relatively low cost, while it also allows to detect *MAP* from formalin-fixed paraffin embedded tissue samples. The latter was demonstrated not only with our bacterial cultures but also with the *MAA*-positive FFPE samples derived from chickens, despite the high genetic similarity of the latter with *MAP*. Finally, the consistency of the results obtained with the said method (B4) by the collaborating laboratories, renders the reaction B4 a most promising method for reliable routine diagnosis of *MAP*.

Considering the technical basis of a PCR reaction, the proposed method with the specific oligonucleotide primers can produce results of equal value (sensitivity - specificity) with samples other that clinical, provided that the DNA extraction procedure produces DNA of similar quality as that mentioned above. The optimum performance of the proposed assay

with the specific oligonucleotide primers has been verified with the detection of MAP from cheese and milk samples that were collected from Greece and the Czech Republic and were evaluated with different PCR assays and with culture.

Description of the Figures and Tables

Table 1 shows the prototype strains of the mycobacterial species that were utilized for the evaluation of the proposed PCR methods.

Table 2 shows the composition of the nucleotide sequences of the primers used.

Table 3 shows the temperature profiles of the PCR reactions B1, B2, B3 and B4.

Table 4 shows comparative PCR results on the FFPE samples with typical lesions of Johne's disease, collected from cattle.

Figure 1 shows representative results from electrophoresis of PCR products of the methods B1, B2, B3 and B4, incorporated to the examination of FFPE samples collected from cattle and chickens.

More specifically,

Lane 1 shows 100bp (Biolabs) DNA ladder.

Lanes 2-4 show DNA products of PCR performed on samples from the small intestine of cattle.

Lane 5 shows DNA products of PCR performed on samples from a mesenteric lymph node of cattle.

Lanes 6-7 show DNA products of PCR assays performed on samples from the ileum of cattle.

Lane 8 shows PCR results produced from a chicken-spleen sample.

Lane 9 shows PCR results produced from a chicken-liver sample.

Lane T shows PCR results produced from negative control samples (no DNA).

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Table 1:
Number of strains of the mycobacterial species that were utilized for the evaluation of the proposed PCR methods.

Number of strains	Species	Source
1	*M. tuberculosis*	RIVM, the Netherlands
1	*M. cannetti*	
1	*M. bovis BCG*	
5	*M. anium*	
1	*M. intracellulare type I*	
1	*M. intracellulare type II*	
1	*M. gordonae I*	
1	*M. fortuitum*	
1	*M. scrofulaceum*	
1	*M. kansasii*	
1	*M. avium paratuberculosis*	
8	*M. tuberculosis*	Hospital of Thoracic Diseases, Greece
3	*M. avium*	
3	*M. gordonae*	
1	*M. celatum*	
1	*M. fortuitum*	
9	*M. avium paratuberculosis*	VRI, Cz Republic

RIVM: National Institute of Public Health and Environment, the Netherlands
VRI: Veterinary Research Institute, Cz Republic

Table 2:
Composition of the oligonucleotide primers.

PCR assay	Primer	Primer composition
B1	IS900-F	ACGCCGCGGGTAGTTA
	IS900-R	GGGGCGTTTGAGGTTTC
B2	s204	TGATCTGGACAATGACGGTTACGGA
	s749	CGCGGCACGGCTCTTGTT
	s345	GCCGCGCTGCTGGAGTTGA
	s535	AGCGTCTTTGGCGTCGGTCTTG
B3	IS01	CTTACCTTTCTTGAAGGGTGTTC
	IS04	GTCGTTAATAACCATGCAGTAATG
	IS02	GTATGGCTTTCATGTGGTTGCT
	IS03	TAACCGTCATTGTCCAGATCAAC
B4	P1N	GCATGGCCCACAGGACGTTGAG
	P2N	CTACAACAAGAGCCGTGCCG
	P3N	GGGTGTGGCGTTTTCCTTCG
	P4N	TCCTGGGCGCTGAGTTCCTC

Table 3

Temperature profiles of the reactions B1, B2, B3 and B4.

	Temperature profile							
	1st stage reaction				2nd stage reaction			
Reaction	Dena/ration (°C/min)	Annealing (°C/min)	Extension (°C/min)	Number of cycles	Dena/ration (°C/min)	Annealing (°C/min)	Extension (°C/min)	Number of Cycles
B1	94/1	63/1	72/1	35	-*	-*	-*	-*
B2	94/1	63/1	72/1	35	94/1	63/1	72/1	35
B3	94/1	62/1	72/1	35	94/1	62/1	72/1	35
B4	94/1	54/1	72/1	16	94/1	67/1	72/1	30

* Reaction B1 is performed in a single stage

Table 4:

PCR results of the methods B1, B2, B3 and B4 on the FFPE samples with typical lesions of Johne 's disease, collected from cattle.

PCR assay	Description	Positive results
B1	PCR	6/10 (60%)
B2	Nested-PCR	9/10 (100%)
B3	Nested-PCR	6/10 (60%)
B4	One tube nested PCR	10/10 (100%)